



PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group Art Unit 1808 :
In re application of :
HITOSHI NAGAOKA : INHIBITOR OF HEPATITIS B
AND HIV ACTIVITY
Serial No. not yet assigned :
Filed June 9, 1994 :
Examiner - I. Marx :

Pittsburgh, Pennsylvania

DECLARATION

Hon. Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

I, Hideo Sawadaishi, declare as follows:

1. I am a citizen of Japan, and residing at Sunny Homes Negishi B
101, Tsutsumidai 83, Noda-shi, Chiba-ken, Japan.

In March, 1975, I was graduated from a prefectural Shimizu Senior
high school, Department of Industrial Chemistry.

Since April, 1975 till 1991, I have been an employee of Noda
Shokkin Kogyo K.K., I was engaged in various research and development
works in Lentinus edodes mycelium.

Since 1991, I have been an employee of NAGAOKA L.E.M. LABORATORY
Co., LTD., and till the present time, I have been engaged in research
and development works in various research and development works in
Lentinus edodes mycelium.

2. I am familiar with the teachings of Japanese Patent Laid-Open Publication No. 01-312980 by Iizuka et al. (Iizuka '980).

3. In order to further support the unexpected results obtained from the extract of the present invention, I carried out the following Experiments I and II.

Experiment I

Lentinus edodes NAGAOKA-391029 strain was prepared by subculturing in an agar plate medium containing 10 g of Malt extract (produced by Oriental Yeast Kogyo K.K.), 1.25 g of powdery yeast extract (produced by Wako Junyaku), 1 g of ammonium tartrate (produced by Wako Junyaku), 500 ml of purified water and 7.5 g of agar. 250 ml of a pre-culture medium containing 20 g of the Malt extract, 2.5 g of the powdery yeast extract, 2 g of the ammonium tartrate and a drop of a silicon defoaming agent in 1 liter of purified water was introduced into 500 ml-Sakaguchi flask and then subjected to autoclaving at 120°C for 30 minutes. After autoclaving, the above-subcultured strain was inoculated into the pre-culture medium in the flask.

Then, the resultant medium was shake-cultured at 20°C for 30 days (shaking width : 7 cm, 100 rpm) to give a pre-culture solution. Separately, a liquid medium containing 160 g of the Malt extract, 20 g of the powdery yeast extract, 16 g of the ammonium tartrate, 8 liters of purified water and 2 ml of the silicon defoaming agent was put into a 10-liter culture bottle provided with a magnetic stirrer, and then subjected to autoclaving at 120 °C for 30 minutes. After autoclaving, a vial of the above pre-culture solution was inoculated into the liquid medium in the bottle, and cultured at 20 °C for 15 days while

introducing sterilized air and slowly stirring with the magnetic stirrer.

The thus cultured liquid was filtered through a filter cloth of about 100-mesh and the filtrate was subjected to lyophilization to give a light brown powdery material having a high moisture absorption (Sample A).

With respect to the Sample A, the anti-Human Immunodeficiency Virus (HIV) effect was measured in the same manner as in Test Examples 1 to 10 of the Preparation Example 1. The results are shown in Table A.

Table A

Test No.	Concentration (μ g/ml)	MT-4		MT-4/HIV	
		Absorbance	Viability (%)	Absorbance	Viability (%)
1	Control	1.321	100.0	0.167	12.6
2	3.9063	1.315	99.5	0.227	17.2
3	7.8125	1.364	103.3	0.226	17.1
4	15.6250	1.310	99.2	0.196	14.8
5	31.2500	1.284	97.2	0.187	14.2
6	62.5000	1.324	100.2	0.218	16.5
7	125.0000	1.297	98.2	0.141	10.7
8	250.0000	1.313	99.4	0.361	27.3
9	500.0000	1.100	88.3	0.403	30.5
10	1000.0000	0.024	1.8	0.024	1.8

As shown in Table A, when the concentration of this Sample A is 125μ g/ml, the viability of the HIV-infected MT-4 cells is as low as 10.7 %.

Experiment II

To 320 g of bagasse (produced by Okinawa Seito K.K.) were added 3.5 liters of purified water, 2 g of cellulase T4 (produced by Amano Seiyaku K.K.) and 2 g of protease P6 (produced by Amano Seiyaku K.K.) to proceed a reaction at 37 °C for 1 hour. After the reaction, the resultant material was filtered through a filter cloth of about 100-mesh and the filtrate was subjected to lyophilization to give a light brown powdery material having a high moisture absorption (Sample B).

With respect to the Sample B, the anti-HIV effect was measured in the same manner as in Test Examples 1 to 10 of the Preparation Example 1. The results are shown in Table B.

Table B

Test No.	Concentration (μ g/ml)	MT-4		MT-4/HIV	
		Absorbance	Viability (%)	Absorbance	Viability (%)
1	Control	1.056	100.0	0.089	8.4
2	3.9063	1.018	96.4	0.096	9.1
3	7.8125	1.125	106.5	0.057	5.4
4	15.6250	1.056	100.0	0.135	12.8
5	31.2500	1.152	109.1	0.115	10.9
6	62.5000	1.258	119.1	0.288	27.3
7	125.0000	0.392	37.1	0.370	35.0
8	250.0000	0.085	8.0	0.087	8.2
9	500.0000	0.027	2.6	0.029	2.7
10	1000.0000	0.030	2.8	0.029	2.7

As shown in Table B, when the concentration of this Sample B is $125\mu\text{ g/ml}$, the viability of the HIV-infected MT-4 cells is as low as 35.0 %.

From the results shown in the Table A and B, and based on my knowledge, I conclude that:

The results of the Experiments I and II show that when the concentration is $125\mu\text{ g/ml}$, the viabilities of the HIV-infected MT-4 cells to the Samples A and B are as low as 10.7 % and 35.0 %, respectively.

By contrast, when the concentration of the pharmaceutical composition for inhibiting Human Immunodeficiency Virus (HIV) of the present invention is $125\mu\text{ g/ml}$, the viability of the HIV-infected MT-4 cells is as high as 71.5 %. Such high viability can be attained by the use of the pharmaceutical composition of the present invention.

4. In order to show the difference between the Lentinus edodes mycelium extract of the present invention and a healthy food of Iizuka '980 reference, I carried out the following Experiments III and IV.

Experiment III

A healthy food was prepared according to the method of Iizuka '980 and the effective components contained therein were analyzed. The analytical data are shown in the following Table C.

Experiment IV

A Lentinus edodes mycelium extract was prepared according to the method of the present invention and the effective components contained therein were analyzed. The analytical data are shown in the following Table C.

Table C

	Analytical method	Ex. III (Iizuka '980)	Ex. IV (the invention)
Water	Heat-drying at ordinary pressure	2.2 %	6.3 %
Protein	Kjeldahl method *	5.4 %	27.0 %
Fiber	Phenneverg Stohmann modification	25.1 %	0.7 %
Lipid	Ether-extraction method	2.1 %	1.7 %
Ash	Direct ashing	8.0 %	21.3 %
Sugar	**	57.2 %	43.0 %

(Note *) Nitrogen/Protein coefficient : 6.25

(Note **) Ash (%) = 100 - (water + protein + fiber + lipid + sugar)

From the results of the above Experiments, and based on my knowledge, I conclude that:

The healthy food of the Iizuka '980 reference is a low protein and fiber rich product, whereas the extract of the present invention is a high protein and low fiber product.

Thus, the healthy food of the Iizuka '980 reference and the extract of the present invention are completely different from each other.

5. I declare further that all statements made herein of my own knowledge are true and that statements made on information and belief are believed to be true; and further that these statements were made

with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

this 4th day of October, 1995

澤田石 英雄
Hideo SAWADAISHI